Haptoglobin genotypes, inflammation status and their associations with hemoglobin levels in stage 4 chronic kidney disease patients with anemia

Reza Alipanah Mogadam[1, 2], Amir Ghorbanihaghjo[2], Hassan Argani[2], Saied Hosseini-Asl[3]

[1] Department of Biochemistry and Clinical Laboratories, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran,
[2] Biotechnology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran,
[3] Medical Genetics Lab., Imam Hospital, Ardabil University of Medical Sciences, Ardabil, Iran.

Abstract

Background and aims: Anemia and increased inflammation are the main causes of mortality in chronic kidney disease (CKD) patients. This study was designed to explore the probable relationship of hemoglobin (Hb) with serum haptoglobin (HP), its genotypes and inflammation statues in CKD stage 4 patients with anemia.

Methods: The subjects of the study was 40 anemic CKD patients on stage 4 [GFR = 15 - 29 ml/min/1.73 m^2]. Serum biochemical factors as well as ferritin, HP and IL-6 levels were measured by standard methods. Haptoglobin genotypes were determent by PCR method and the accuracy of genotyping was confirmed by sequencing amplified fragments and RFLP analysis.

Results: Results showed that although HP2-2 was the most common genotype (%72.5), no significant correlations between HP genotypes with Hb and HP levels were found ($p= 0.49$, $p= 0.32$, respectively). Significant negative correlation between Hb level with IL-6 and hs-CRP levels ($p< 0.001$ and $p< 0.05$, respectively) and a significant positive correlation between Hb and ferritin levels was found ($p< 0.05$).

Conclusion: The results suggest that HP genotype and serum HP levels have no significant effect on hemoglobin level in CKD stage 4 patients. However, Hb level is associated with inflammation and albumin status.

Keywords: Haptoglobin genotype, CKD, hemoglobin, inflammation.

Introduction

The incidence and prevalence of kidney disease worldwide has risen markedly in the past decade [1]. It has been shown that, the incidence of chronic kidney disease (CKD) in stages 3 to 5, in Iran is about 14.9% [2]. Chronic kidney disease is the most commonly defined by a reduction in kidney function [3]. The anemia is a common complication in patients with CKD and correlates significantly with the morbidity and mortality in these patients [4].

The main causes of anemia in CKD are reduced production of erythropoietin in the kidneys and erythropoietin resistance [5, 6]. Inflammation also has an important role in CKD complications [7]. Inflammatory factors such as IL-6 inhibit erythropoietin production, impair the growth of erythroblasts, decreases RBC survival and promote death of immature erythroblasts [5, 7]. Studies shown that patients who had higher plasma levels of inflammatory markers shows lower hemoglobin levels than those with lower level of inflammation [8]. On the other hand, Inflammation is an important factor associated with erythropoietin resistance and the occurrence of anemia in patients with CKD [9]. Inflammation also limits the availability of iron for erythropoiesis and expands the erythropoietin resistance [10]. One of the major inducible cytokine inflammatory proteins that participate in hemoglobin and iron metabolism called haptoglobin (HP) [11]. Haptoglobin is the major hemoglobin-binding protein in the plasma of most vertebrates and all mammals [12]. Haptoglobin is synthesized primarily via hepatocytes

and expressed by a genetic polymorphism as three major genotypes HP1-1, HP2-1, HP2-2 [13]. Both HP 1 and HP 2 alleles have been found in all population, although their frequencies vary considerably among them [14]. The primary physiological function of HP has been described in terms of its interaction with free Hb and prevents hemoglobin deposition in the glomeruli and proximal tubule cells of the kidney and prevents body iron loss [15]. In CKD patients, uremic toxins can shorten the erythrocyte life span leading to hemolysis and contribute to renal anemia [5, 16]. It has been shown that the different HP genotypes have different effects on the rate of uptake and removal of hemoglobin released from erythrocytes [17]. The other possible function of HP is the participation in iron metabolism as HP affects the regulation of the ferroportin (the major transporter of iron) expression and iron transferring from duodenal mucosa to the plasma [18]. The main part of exported iron from duodenal mucosa by the ferroportin is used to sustain de novo erythropoiesis [19]. It has been shown that haptoglobin-null mice export significantly more iron from the duodenal mucosa to the plasma compared to the control counterparts [18]. Few studies have shown the effects of serum HP concentration and its genotypes on body iron status [19, 20] and their association with hemoglobin level is not fully elucidated. We hypothesized that HP can influence on hemoglobin levels, therefore this study aimed to evaluate the probable relationship between hemoglobin levels with HP serum level; its genotypes and inflammation in CKD stage 4 patients with anemia.

Materials and methods

Patients and Study Design

The study was performed in the Department of Biochemistry of Tabriz University of Medical Sciences (TUMS). The ethics committee of TUMS approved the study. Recruitment of the patients occurred from Mar 2011 through Oct 2012. Forty patients (15 males and 25 females) were included in the study. Anemic adult patients (≥18 yr) who had CKD stage 4 with GFR = 15 - 29 ml/min/1.73 m^2 were included in the study. Anemia was defined as hemoglobin levels of less than 13 g/dl for men and postmenopausal women and less than 12 g/dl for premenopausal women [21]. Subjects with transfusion within past 6 months, myocardial infarction history within past 3 months, surgical history within past 3 months, malignancy, diastolic blood pressure greater than 100 mm/hg, uncontrolled diabetes, severe hyperparathyroidism (PTH > 800 pg/ml) and consumers of drugs such as angiotensin receptor blockers and angiotensin-converting enzyme inhibitors were excluded from the study.

Biochemical assays

Blood samples were obtained after an overnight fast and hemoglobin and hematocrit levels were measured by blood cell counter (Sysmex KX-21N, Japane). Separated Serum and whole blood were kept frozen at –80°C until analysis. The serum was analyzed using an auto-analyzer (BT3000, Italy) and manufacture's reagent kits for Mg, hs-CRP, TIBC, Fe, Ca, P, Urea, Cr, Alb and Total protein. Serum HP and ferritin levels were assayed by standard enzyme linked immunosorbent assay (ELISA) kits (Cusabio and DiaPlus, China, USA, respectively). Serum concentrations of IL-6 were measured using human ELISA kits according to the manufacturer's instructions (Bender Med Systems, Vienna, Austria). These biochemical factors were assayed using fully automated ELISA analyzer (TKA Teknolabo Bizet, Italy). The PTH Chemiluminescent Activity Assay kit is designed to measure PTH activity (Liaison, USA) and GFR was calculated by the Cockcroft-Gault formula [22].

Haptoglobin genotyping

Genomic DNA was extracted from peripheral blood leukocytes using the 5-prime ArchivePure DNA blood kit (Ipsogen, Germany). The primer sequences used for amplification of HP1 and HP2 alleles were previously described [23] and listed in Table1. The primers A and B were designed to genotyping the HP alleles via amplification of a 1757-bp length of fragment responsible for HP1 allele and a 3481-bp length of HP2 allele- specific sequence [23]. Detecting the HP2 allele was carried out by amplification of a 349-bp fragment with primer C and D, as well. The 20µL reactions contained 2 U of Taq polymerase (Fermentas Co, Canada), 1–100 ng of DNA, and 200µM dNTPs mix;

PCR buffer was used as suggested by the supplier (Fermentas Co, Canada) with no supplements added. After initial denaturation at 95 °C for 5 min, the two-step thermo cycling procedure consisted of denaturation at 94 °C for 45 second and annealing at 62 °C for 1 min (in the presence of primers A, B, C, and D), and extension at 72 °C for 1 min, repeated for 35 cycles, and followed by a final extension at 72 °C for 7 min. The thermocycler instrument used was Veriti 96 well (Applied Biosystems, USA). For genotype assignments, the PCR products were separated in 1% agarose gel (Sigma-Aldrich Co, USA).

The accuracy of genotyping was confirmed by RFLP analysis. The 1757-bp HP1 allele–specific fragment is digested by MlsI enzyme (Fermentas Co, Canada) to two 551- and 1206-bp fragments. Presence of HP2 allele could be detected by digesting the 349-bp amplified product with DraI restriction enzyme (Fermentas Co, Canada) which produced two 193- and 156-bp fragments. After evaluating the accuracy of amplification by two separated primer pairs and PCR-RFLP, they were analyzed by sequencing and blasting them for approaching confidence of genotyping.

Statistical analysis

The collected data were analyzed by statistical methods such as tables and Chi-square, analysis of variance and Pearson's Correlation Coefficient in SPSS software version 16. For all tests, a p value < 0.05 was considered statistically significant.

Results

Clinical features of study subjects were shown in Table 2. Results of PCR genotyping and RFLP for alleles of HP1 and HP2 were displayed in Figure 1. The mean age of all patients was 55.68 ± 14.4 years old. As indicated in table 3, of 40 cases, 15 (37.5%) were male and 25 (62.5%) female. Our results showed that HP2-2 was the most common genotype detected; however patients with HP1-2 were predominantly male. The patients were divided into low (6-9 g/dl) and high hemoglobin levels (9-13 g/dl). The results showed that 9 (22.5%) and 31(77.5%) of subjects had serum low and high hemoglobin levels, respectively. Although, the low levels of hemoglobin were seen in subjects with HP1-2 genotype, but there was no significant relationship between hemoglobin

Table 1. The sequences of the primers used for PCR amplification of HP1 and HP2 alleles

Primer	Sequence	Specific allele	Fragment size
A	'-GAGGGGAGCTTGCCTTTCCATTG-3'	HP1 & HP2	HP1:1757-bp HP2:3481-bp
B	5'-GAGATTTTTGAGCCCTGGCTGGT-3'		
C	5'-CCTGCCTCGTATTAACTGCACCAT-3'	HP2	349-bp
	5'-CCGAGTGCTCCACATAGCCATGT-3'		

Table 2. Clinical features of the study subjects

Variable	Mean±SE	Variable	Mean±SE	Variable	Mean±SE
Urea (mg/dl)	126.38 ± 7.12	P (mg/dl)	4.36 ± 0.12	IL-6 (pg/ml)	0.59 ±3.39
Cr (mg/dl)	3.66 ± 0.15	Hb (g/dl)	10.01 ± 0.24	hs-CRP (mg/l)	10.54 ± 1.89
TIBC (µg/dl)	295.05 ± 8.18	HCT (%)	31.15 ± 0.69	Ferritin (µg/l)	163.83 ± 24.69
Fe (µg/dl)	63.5 ± 4.64	HP (mg/dl)	62.45 ± 9.31	PTH (pg/ml)	111.93 ± 13.35
Ca (mg/dl)	9.03 ± 0.11	Alb (g/dl)	4.21 ± 0.11	GFR (ml/min/1.73 m^2)	21.51 ± 0.74
Mg (mg/dl)	2.01 ± 0.04	Total protein(g/dl)	8.11 ± 0.17	Weight (kg)	72.4 ± 1.73

Cr= Creatinine, TIBC= Total Iron Binding Capacity, Fe= Iron, Ca= Calcium, Mg= Magnesium, P= Phosphorus, HCT= Hematocrit, HP=Haptoglobin, Alb=Albumin, IL-6= interleukin-6, PTH= parathyroid hormone, GFR= Glomerular filtration rate

Table 3. Distribution of gender and the level of hemoglobin between the different HP genotypes

Genotype	Total frequency *	Male *	Female *	Hemoglobin level (g/dl)±SE
HP1-1	12.5	20	80	10.54 ± 0.44
HP1-2	15	66.7	33.3	9.35 ± 1.04
HP2-2	72.5	34.5	65.5	10.05 ± 0.26

**The results are noted as percentage*

level and HP genotype (p= 0.49). There was no significant relationship between HP genotype and serum HP level (p= 0.32). No meaningful relation was observed between hemoglobin level and serum HP level (p=0.8); however there was a significant positive correlation between hemoglobin level with ferritin and albumin levels. We found a significant negative relationship among hemoglobin level with IL-6 and hs-CRP (p< 0.001 and p< 0.05), respectively. There was a significant positive correlation between serum magnesium and hemoglobin level (p<0.05).

Discussion

CKD is a worldwide public health problem [24]. Anemia is one of the main problems of CKD and occurs especially in the final stages of the disease [25]. It has been shown that the anemia is associated with lower quality of life and a higher risk of adverse outcomes including cardiovascular disease and finally death [26]. HP is one of the plasma proteins that may affect hemoglobin level and the anemia [27]. In the present study we did not find a significant relationship among hemoglobin level with different HP genotypes and serum HP level. In contrast with our findings, some other studies in animal model, thalasemic patients and patients with bone marrow failure syndromes have shown a significant positive relationship between hemoglobin level with HP genotype and serum HP level [19, 28, 29]. In other studies Atkinson et al found a positive significant relationship between HP genotype and hemoglobin level in healthy subjects [30, 31]. This inconsistency may be due to factors such as inflammation, hepatic dysfunction, uremic toxins and renal failure in CKD patients that can change hemoglobin and HP levels [16, 25, 32, 33]. Several studies have indicated that there exists inflammation in CKD patients [32]; it has been shown that inflammation increases plasma HP concentration up to 3- to 8-fold by increased levels of pro-inflammatory cytokines, e.g. IL-6 [34]. Also, inflammatory mediators such as interleukins and tissue necrosis factor, blunt the effect of erythropoietin on the bone marrow, suppress the growth of erythroid colony-forming units (CFU-E), downregulate the expression of erythropoietin receptors on erythroid progenitors and disrupt iron recycling by blocking its release from reticuloendothelial cells [24]. Thus, in CKD patient's chronic and severe inflammation [32] may result in disruption of the balance between hemoglobin and HP levels. Reduction in levels of HP through losing from kidneys, decreased synthesis in liver and reduced erythrocyte life span due to uremic toxins can exacerbate this imbalance [5, 33, 35]. In the best of our knowledge there are no reports on relationship among hemoglobin level with different HP genotypes and serum HP level in CKD stage 4 and it needs further investigation to be elucidated.

We observed significant inverse association among hemoglobin level with hs-CRP and IL-6. The prevalence of inflammation in uremic pati-

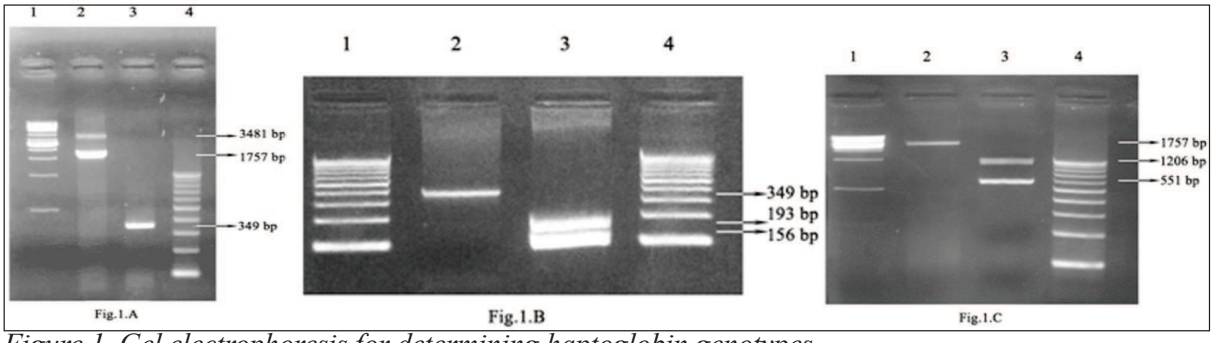

Figure 1. Gel electrophoresis for determining haptoglobin genotypes

(A): Genotyping by PCR primers A-B and C-D. Lane 1, 1 Kb DNA ladder; Lane 2, HP1-2 (amplified fragment with 3481 bp length for genotype 2 and 1757 bp length fragment for genotype 2); Lane 3, 349 bp length fragment for showing presence of allele 2, Lane 4, 100 bp DNA ladder. Using primers pair C and D confirmed the accuracy of genotyping by the A and B pair.

(B): Digesting the allele-2 related amplified fragments by DraI for approaching the accuracy of genotyping. Lane 1 & 4, 100 bp DNA ladder. Lane 2, uncut fragment. Lane 3, cut fragments.

(C): Confirming the accuracy of allele-1 related fragment by MlsI digestion. Lane 1, 1Kbp DNA ladder. Lane 2, uncut fragment. Lane 3, cut fragments. Lane 4, 100 bp DNA ladder.

ents is high [36]. Several studies have revealed a strong relationship between anemia and chronic inflammation, and suggest that chronic inflammation is associated with lower hemoglobin concentrations even in the early stages of CKD [36-38]. Our findings are in agreement with the findings of other studies showed that an inverse relationship between hemoglobin level and inflammatory factors such as hs-CRP and IL-6 [39, 40]. Other independent support for this hypothesis is provided by the observation that end stage renal disease patients with elevated blood levels of IL-6 are more anemic, and this anemia is correlated with increased cytokine levels [41]. Evidence suggests several mechanisms by which inflammation may affect the development of anemia [9]. Elevated levels of inflammatory cytokines enhanced oxidative stress and alterations in iron metabolism, conditions associated with inflammatory states, may be implicated in the development of anemia [37, 42]. However, inflammation-induced anemia and resistance to erythropoietin are common features in patients with advanced CKD [24]. In dialysis patients, high CRP levels are associated with low Hb levels and/or erythropoietin resistance [36]. The increased levels of inflammatory cytokines such as IL-6 lead to impaired ability of RBC progenitors to respond to erythropoietin, shortened red blood cell survival and decreased the Hb concentration [7, 36]. Hence, chronic inflammation is an important cause for decreased in Hb level in CKD stage 4.

We also found a significant positive relationship between hemoglobin and ferritin levels which is similar to previous findings [43]. Saigo et al in myelodysplastic syndrome [44] and Locatelli et al in chronic hemodialysis patients [45] showed a significant negative relationship between ferritin and hemoglobin levels, quite the opposite to our findings. These findings contrast with those of Cazzola et al who did not found a significant relationship between hemoglobin and ferritin levels in systemic-onset juvenile chronic arthritis [46]. Availability of iron is key for optimal erythropoiesis and serum ferritin levels in healthy individuals reflect the magnitude of the mobilizable body iron stores, i.e., the iron that is available for the synthesis of hemoglobin in red blood cells [47]. It has been reported that the hemoglobin and ferritin biomarkers were influenced by inflammation [48]. Serum ferritin is an acute phase protein and increases two to fourfold in response to inflammation [36]. Studies in cellular and animal models indicate that inflammatory cytokines such as IL-6 can enhance ferritin synthesis and increase hepatic uptake of serum iron [46]. In turn, increased ferritin expression results in reticuloendothelial iron block and impairs iron absorption [46]. Inflammation leads to blockage in iron utilization and "anemia of chronic disease [49]. Studies have shown that there is a significant association between serum ferritin level and the degree of severity of anemia [50, 51]. More studies are needed to correlate ferritin and hemoglobin level in patients CKD stage 4 with anemia.

In our study there was a significant positive relationship between hemoglobin and albumin levels [41]. Significant positive relationship between hemoglobin and albumin has been shown also in other studies [41, 52]. This finding suggests that anemia is also associated with both cytokine-dependent inflammation and nutritional status of patients.

Conclusion

In conclusion, our study suggests that HP genotype and serum HP levels have no effect on hemoglobin level in CKD stage 4. However, Hb level is associated with inflammation and albumin status. This finding suggests that anemia in these patients is associated with both cytokine-dependent inflammation and nutritional status of patients.

Acknowledgments

The authors gratefully acknowledge the Biotechnology Research Center, Tabriz University of Medical Sciences, for their financial supports and patients participated in this study.

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Corresponding Author
Amir Ghorbanihaghjo,
Biotechnology Research Center,
Tabriz University of Medical Sciences,
Tabriz,
Iran,
E-mail: ghorbaniamir@hotmail.com